ELSEVIER

Journal of Cereal Science 44 (2006) 353–367

Journal of CEREAL SCIENCE

www.elsevier.com/locate/yjcrs

Biochemical and genetic characterization of wheat (*Triticum* spp.) kernel polyphenol oxidases ☆

James V. Anderson[a,*], E. Patrick Fuerst[b,d], William J. Hurkman[c], William H. Vensel[c], Craig F. Morris[d]

[a] *USDA-ARS, Plant Science Research, 1605 Albrecht Blvd., Fargo, ND 58105-5674, USA*
[b] *Department of Crop & Soil Sciences, Washington State University, Pullman, WA 99164-6394, USA*
[c] *USDA-ARS, Western Regional Research Center, Albany, CA 94710, USA*
[d] *USDA-ARS, Western Wheat Quality Laboratory, Pullman, WA 99164-6394, USA*

Received 26 March 2006; received in revised form 27 June 2006; accepted 27 June 2006

Abstract

Genetic variability of polyphenol oxidase (PPO) activity was evaluated in hexaploid, tetraploid, and aneuploid wheat (*Triticum* spp.). A DNA probe to kernel-specific PPO hybridized to PPO clones from an immature wheat kernel cDNA library but not to clones from a seedling cDNA library. Recombinant protein and antibody were developed from the kernel-specific PPO. PPO transcript and antigenic proteins were most abundant during kernel development and decreased rapidly at physiological maturity. PPO proteins were more abundant in immature kernels, but PPO activity was greater in mature kernels. Wheat kernel antigenic protein bands of ~58, 60, 62, and 75 kD were identified; immunoprecipitated proteins of ~60 and ~62 kD were confirmed to be PPOs by LC-MS/MS. The 2D chromosome was implicated as a major locus of PPO activity in cultivar (cv.) 'Chinese Spring' based on antigenic staining intensity of ~60–62 kD proteins and kernel PPO activity in aneuploid lines. The ~58 kD protein was not well-correlated with kernel PPO activity, while the ~75 kD protein was likely a PPO precursor. Tropolone completely inhibited PPO activity extracted from mature kernels but not from immature kernels. Total PPO activity in mature kernels is thus a function of specific PPO isoforms present, their abundance, and activation levels.

Keywords: Polyphenol oxidase; Wheat; *Triticum aestivum*; *Triticum turgidum* ssp. *durum*; Aneuploid; Wheat quality; Darkening; Discoloration

1. Introduction

Polyphenol oxidase (PPO) has been implicated as a major causative agent of darkening in raw Asian noodles and other wheat products (Morris et al., 2000), in raw sugar (Vickers et al., 2005), and in many fruits and vegetables (Whitaker and Lee, 1995). In the Asian Pacific Rim, consumer acceptability of noodles (primarily yellow alkaline and white salted) prepared from imported wheat is heavily dependent on end-product color (Morris et al., 2000). Competition for this market among world wheat exporters has a direct impact on US wheat exports and consequently US agriculture. Improved methods for conducting rapid whole-kernel PPO assays (AACC, 2000; Anderson and Morris, 2001) have helped to eliminate high PPO wheat breeding lines but have done little to address the biochemical and genetic basis for variability in PPO activity.

PPO (tyrosinase) was first reported in wheat bran (Bertrand and Muttermilch, 1907), and has been extensively studied in other plant systems (Steffens et al., 1994).

Abbreviations: cv., cultivar; DPA, days post-anthesis; L-DOPA, 3, 4-dihydroxy-L-phenylalanine; MOPS, 3(*N*-morpholino) propanesulfonic acid; PPO, polyphenol oxidase; SDS-PAGE, sodium dodecylsulfate-polyacrylamide agarose gel electrophoresis; Tris, Tris(hydroxyethyl)amino-ethane; TBST, Tris-buffered saline with Tween 20

☆ Mention of trademark or proprietary products does not constitute a guarantee or warranty of a product by the US Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable. This article is in the US Public Domain and is not copyrightable.

*Corresponding author. Tel.: +1 701 239 1263; fax: +1 701 239 1252.
E-mail address: andersjv@fargo.ars.usda.gov (J.V. Anderson).

0733-5210/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.jcs.2006.06.008

However, it is only recently that (1) diphenol (3,4-dihydroxy-L-phenylalanine—L-DOPA) oxidation in wheat has been specifically attributed to PPO based upon sensitivity to PPO inhibitors (Fuerst et al., 2006b), (2) a wheat PPO protein has been purified (Anderson and Morris, 2003b), and (3) wheat PPO genetic clones have been reported (Anderson and Morris, 2003a; Demeke and Morris, 2002; Jukanti et al., 2004; Morris et al., 2002). Consequently, there is still much to be learned about the genetics and biochemistry of wheat PPOs.

Most plant PPOs (EC 1.14.18.1; monophenol monooxygenase [tyrosinase and cresolase] or EC 1.10.3.2; diphenol oxygen oxidoreductase [catecholase]) appear to belong to a nuclear-encoded, multigene family (Steffens et al., 1994). For example, tomato contains seven PPO genes classified into three structural groups (Newman et al., 1993), potato contains six PPO genes which exhibit specific temporal and spatial patterns of expression (Hunt et al., 1993; Thygesen et al., 1995), and red clover has at least three PPO genes that are tissue-specific and differentially expressed (Sullivan et al., 2004). Numerous other reports support the existence of a plant multigene PPO family. Sequence data indicate that PPO genes encode two conserved Cu-binding domains and a transit peptide required for import into the plastid lumen (Steffens et al., 1994; Sullivan et al., 2004); one exception is an aureusidin synthase, a PPO homolog of snapdragon, which lacks a transit peptide and is most likely located in vacuoles (Nakayama et al., 2000). Unprocessed PPOs (precursor forms containing the chloroplast lumen transit peptide) are generally reported to be 60–75 kD and the processed PPOs (referred to as the mature, active form devoid of the chloroplast lumen transit peptide) are generally 45–69 kD. Latent forms of PPO complicate measurement of total enzyme activity; aging, salts, detergents, solvents, protease treatment, and urea have been reported to activate latent forms of PPO (Okot-Kotber et al., 2001; Steffens et al., 1994).

In wheat, two distinct clusters of PPO genes were characterized based upon re-sequencing of clones representing wheat ESTs (Jukanti et al., 2004). Each cluster contained three distinct forms of PPO, indicating the presence of at least six genes. The two clusters were associated with genes expressed in either kernel or non-kernel tissues. Southern blots also indicated the likelihood of three homoeologous PPO genes (Demeke and Morris, 2002) for what we now believe to be the non-kernel PPOs. QTL analysis indicated that PPO activity was associated with chromosomes 2A, 2B, 3D, and 6B (Demeke et al., 2001). In hexaploid wheat, the 2D chromosome was associated with enhanced levels of kernel PPO activity (Anderson and Morris, 2001) and Mares and Campbell (2001) further demonstrated that QTLs for noodle darkening and PPO activity coincided on the 2D chromosome. In tetraploid wheat (*T. turgidum* ssp. *durum* [Desf.] Husn.), which lacks the D genome, high PPO activity mapped to the long arm of chromosome 2A (Raman et al., 2005; Simeone et al., 2002).

The objective of this study was to characterize the molecular and biochemical basis for the complex genetic variability that has long been reported for kernel PPO activity among different wheat cultivars. In this study, we have (1) characterized a full-length cDNA clone for a putative wheat kernel-localized PPO, 2) produced a recombinant PPO protein and confirmed its identity by LC-MS/MS, (3) developed a polyclonal antibody to the recombinant PPO protein, (4) monitored PPO transcript and antigenic protein levels in developing kernels of cvs. having different levels of PPO activity in mature kernels, (5) compared antigenic wheat PPO protein in cvs. having high or low PPO activity and in aneuploid wheat lines, and (6) evaluated sensitivity of L-DOPA oxidation to tropolone in immature and mature wheat kernel extracts. Data obtained from this study, although linking the 2D chromosome of hexaploid wheat with increased kernel PPO activity, suggest that other factors also influence kernel PPO activity and possibly contribute to discoloration of wheat products.

2. Experimental

2.1. Plant material

Kernels used for this study were obtained from the USDA-ARS, Western Wheat Quality Lab, Pullman, WA or from the USDA-ARS Cereals Unit, Fargo, ND. Kernels from hexaploid cvs. (Penawawa, ID377s, Klasic, and Butte 86), and a tetraploid cv. (Langdon) were germinated in Sunshine mix #1 (Sun Gro Horticultural Distribution Inc., Bellevue, WA), watered daily, and maintained at 25 °C under a 16:8 day:night photoperiod. Leaf tissue and kernels collected at various time points were immediately frozen in liquid N_2 and stored at −80 °C. Langdon substitution lines (Joppa and Williams, 1988) are abbreviated, for example, as N2A/D2D for nullisomic 2A/disomic 2D. Chinese Spring nullisomic–tetrasomic lines (Gill et al., 1991) are abbreviated, for example, as N2D/T2A for nullisomic 2D/tetrasomic 2A. Both the Langdon and Chinese Spring substitution lines were provided by Dr. Justin Faris, USDA/ARS.

2.2. Isolation of cDNA clones for wheat PPO

Wheat DNA probes to the coding regions of putative kernel-specific and non-kernel-specific cDNA clones (GenBank accessions BT009357 and AF507945, respectively) were [^{32}P]-labeled as previously described (Anderson and Davis, 2004) and used as probes to screen a cDNA library constructed from developing grain of wheat cv. Butte 86 (TAcDNA0025 Lot#1.1.L, TA059E1X) and a cold-stressed seedling subtracted library of cv. Chinese Spring (libraries were obtained from Dr. Timothy Close, University of California, Riverside). Clones showing hybridization after two 15-min stringency washes at 55 °C in 1 × SSC and 0.1% sodium dodecylsulfate (SDS) (see

Sambrook et al., 1989) were isolated and the inserts were fully sequenced. Sequence data obtained from isolated cDNA were analyzed using GenBank BlastX (Altschul et al., 1997) to obtain best-match sequence identities. Sequence alignments were accomplished with the MegAlign program of Lasergene 6.0 (DNAstar Inc., Madison, WI).

2.3. Expression analysis of PPO

RNA was extracted from wheat tissue using the procedure of Chang et al. (1993) as described by Anderson and Davis (2004). Total RNA (10 μg) was separated on 1% denaturing agarose gels and blotted onto a positively charged nylon membrane (Hybond-N, Amersham Pharmacia Biotech) using standard protocols (Sambrook et al., 1989). Northern blot hybridizations using [^{32}P]-labeled wheat PPO cDNA (accession BT009357) were conducted as previously described by Anderson et al. (2004). Quantification of hybridizing counts on RNA blots was accomplished using a Packard Instant Imager (Packard Instrument Co., Downers Grove, IL).

2.4. Development of recombinant wheat PPO and antibodies

Recombinant wheat PPO for accession BT009357 was produced by the Roche Protein Expression Group (Roche Diagnostics Corp., Indianapolis, IN). The full-length coding sequence, including sequence for a C-terminal 6X His-tag (GenBank nucleotide accession DQ217371; protein accession ABA62017), was ligated in-frame into a pIVEX vector and expressed in *E. coli* using the arabinose inducible system. Recombinant protein was purified by Ni affinity and size exclusion chromatography; amino acid sequence was confirmed by tryptic digestion and MALDI-MS analysis. Polyclonal antibody was obtained from a final bleed of rabbits injected with the recombinant wheat PPO protein (Harlan Bioproducts for Science Inc., Madison, WI).

2.5. Protein extraction

Wheat kernels and leaf tissue samples were ground to a fine powder in liquid N_2 using a mortar and pestle and extracted with 50 mM 3(*N*-morpholino) propanesulfonic acid (MOPS) buffer (pH 6.5), 100 mM KCl, 0.2 mM Pefabloc, 0.1% SDS, and 1.0% polyvinylpyrrolidone (4 ml g^{-1} tissue). NP-40 (0.2%) was added to the final solution. After 10 min incubation on ice, the extracts were centrifuged at 20,000 *g* for 15 min at 4 °C. Total protein concentration was determined using the Bradford procedure according to the manufacturer's instructions (BioRad, Hercules, CA).

2.6. Kernel PPO activity assays

Whole-kernel PPO activity was measured after 1 h incubation as previously described (AACC, 2000; Anderson and Morris, 2001). PPO activity staining of 10% polyacrylamide agarose gel electrophoresis (PAGE) gels containing 0.1% SDS (referred to as 10% SDS-PAGE) and PPO kinetic assays were conducted as previously described (Anderson and Morris, 2003b). All assays were conducted with 10 mM L-DOPA in 50 mM MOPS (pH 6.5). Activity gels were pre-treated with ±1 mM tropolone (Aldrich, Milwaukee, WI) in 50 mM MOPS (pH 6.5) for 15 min prior to incubation in 10 mM L-DOPA ± 1 mM tropolone for 1–2 h. Kinetic assays were conducted in 96-well microtiter plates (Nalge Nunc International, Rochester, NY) at ambient temperature (21 °C) to evaluate the effects of tropolone and heat treatment on the rate of L-DOPA oxidation. Kernel extract (5–10 μl) was placed in wells containing 100 μl of 50 mM MOPS buffer (pH 6.5). Extract was either untreated, pre-incubated with 2 mM tropolone for 10 min (1 mM final assay concentration), or heat-denatured by incubating at 100 °C for 5 min. Assays were initiated by adding 100 μl of 20 mM L-DOPA (10 mM final concentration) in 50 mM MOPS (pH 6.5) and absorbance at 490 nm was measured with a microplate reader (BioRad Model 3550; BioRad Inc.). Specific activity is reported as the initial linear rate, measured over the first 2 min, while total activity is reported as the activity measured from the same assay after 30 min.

2.7. Immunoprecipitation of PPO

Prior to immunoprecipitation, crude protein extracts were fractionated with ammonium sulfate as previously described (Anderson and Morris, 2003b). Protein from a 30–70% ammonium sulfate cut was dialyzed overnight with 50 mM Tris(hydroxyethyl)aminoethane (Tris) (pH 7.5) at 4 °C. To compare PPO from different wheat cvs., equal protein content (1–2 mg) in 700 μl total volume was incubated with 5 μl of PPO antibody in 1.7-ml microcentrifuge tubes for a minimum of 2 h at 4 °C with rotation. Antibodies were recovered by incubating with 30 μl of Protein G Sepharose 4B (Amersham Pharmacia Biotech) at 4 °C for a minimum of 2 h with rotation. After centrifugation at 5,000 *g* for 2 min, the supernatant was removed and the Protein G complex was washed with 500 μl of Tris-buffered saline [25 mM Tris base (pH 8.0), 138 mM NaCl, and 2.7 mM KCl] with 0.05% Tween 20 (TBST). The washing procedure was repeated 3 times. SDS-PAGE sample buffer (50 μL; BioRad) containing 5% β-mercaptoethanol was added to the final pellet of the Protein G complex and boiled for 5 min prior to separating 25 μl of the sample by SDS-PAGE.

2.8. SDS-PAGE and Western analyses

Protein extracts were separated on either 7.5% or 10% SDS-PAGE as previously described (Anderson and Davis, 2004). Proteins were detected by either Commassie Blue staining (BioRad) or transblotting to PVDF membrane (BioRad). Transblots were incubated in blocking buffer

(TBST and 5% bovine serum albumin) for at least 1 h prior to incubation in a 1:10,000 dilution of wheat PPO antibody for at least 1 h. Blots were washed 4×15 min with TBST prior to incubation with a 1:10,000 dilution of goat anti-rabbit conjugated alkaline phosphatase (Sigma) secondary antibody for at least 1 h. Blots were washed an additional 4×15 min in TBST, followed by 2×5 min in dd H_2O, and developed by addition of NBT and BCIP (Sigma). Optical density and estimate of MW range of antigenic proteins was determined with a Fluor-S imager (BioRad Inc.) and Quantity One imaging software.

2.9. LC-MS/MS of tryptic peptides of proteins

Proteomic analysis procedures (Vensel et al., 2005) were applied to the structural characterization of putative PPO proteins that had been immunoprecipitated. Tryptic peptides were generated from SDS gel bands using an automatic digestion robot (DigestPro, Intavis, Langenfeld, Germany). Briefly, in-gel Coomassie-stained bands were destained, reduced, alkylated with iodoacetamide, and digested with trypsin. The peptides were eluted from the gel pieces and collected in a 96-well plate in preparation for analysis using a QSTAR *PULSAR i* (Applied Biosystems/MDS Sciex, Toronto, Canada) quadrupole time-of-flight mass spectrometer (Vensel et al., 2005). Data were acquired using the IDA acquisition mode of Analyst QS version 1 software. Data acquisition files created by Analyst QS were converted to text files (Boehm et al., 2004). Data files were submitted to a locally installed version (2005.6.1.2) of X! Tandem (Craig and Beavis, 2004; Fenyo and Beavis, 2003) which was used to match MS/MS fragmentation data to peptide sequences of all plant proteins in the NCBI non-redundant database (May 6, 2005). The Global Proteome Machine software (http://www.thegpm.org/) was used to analyze and display the results.

3. Results

3.1. Identification of cDNAs for putative kernel and non-kernel wheat PPOs

A search of GenBank, using sequence to a partial genomic PPO clone amplified from hexaploid (cv. Penawawa) wheat (Anderson and Morris, 2003a), identified a previously uncharacterized wheat (cv. Stephens) nucleotide sequence (GenBank accession BT009357) with ~71% amino acid identity over similar coding regions (data not shown). BlastX results indicated that accession BT009357 had the best sequence homology to other known plant PPOs and contained a full-length PPO coding sequence (see Fig. 1, protein accession ABA62017).

PPO accession AF507945 from hexaploid wheat cv. Penawawa (Demeke and Morris, 2002) was classified as a non-kernel-localized wheat PPO (Jukanti et al., 2004). Alignment of the amino acid sequences obtained from the open reading frames of BT009357 and AF507945 (GenBank protein accessions ABA62017 and AAM33417, respectively) compared with the previously classified wheat cDNAs reported by Jukanti et al. (2004) as kernel- (accessions AAT06525, AAT06526, AAT06527) or non-kernel- (accessions AAT06523 and AAT06524) specific PPOs are shown in Fig. 1. The alignments of amino acid sequences indicate that protein accessions ABA62017 and AAM33417 clearly represent two differing classes of wheat PPOs and are referred to as either kernel- or non-kernel-specific, respectively, throughout this report.

3.2. Hybridization with tissue-specific cDNA libraries

To further confirm the tissue specificity of wheat PPOs, probes to the coding regions of the putative kernel- and non-kernel-specific clones were used to screen wheat cDNA libraries constructed from developing kernels (cv. Butte 86) or from seedlings (cv. Chinese Spring). The kernel-specific PPO probe from BT009357 hybridized with clones from the developing kernel library but not to clones from the seedling library (data not shown). Probing of the same two cDNA libraries with non-kernel-specific PPO probe from AF507945 resulted in no identifiable hybridizations with either library (data not shown). A nearly full-length cDNA isolated from the developing kernel library had ~86.6% and ~80.6% identity to wheat PPO BT009357 at the nucleotide level (data not shown) and translated amino acid levels (Fig. 1), respectively, and was deposited in GenBank (nucleotide accession AY515506, protein accession AAS00454). The predicted lumen cleavage site shown in Fig. 1 for protein accessions ABA62017 and AAS00454 was based on data reported for PPOs of clover (Sullivan et al., 2004).

3.3. Expression of wheat PPO transcript

Wheat PPO transcript was monitored in developing wheat kernels using kernel-specific BT009357 [^{32}P]-labeled DNA probe. Kernels from wheat cvs. Penawawa and Klasic, which have high kernel PPO activity (Anderson and Morris, 2001), also had higher PPO transcript levels (Fig. 2A) than cvs. with lower kernel PPO activity (ID377s and Langdon). PPO transcript levels were greatest during early kernel development and gradually declined as kernels matured in all cvs. evaluated. No PPO transcript hybridization was observed in young leaf tissue collected from two hexaploid wheat cvs. (Penawawa and Butte 86) when probed with the putative kernel-specific wheat PPO (Fig. 2B). The non-kernel-specific AF507945 DNA probe did not hybridize to transcripts from either wheat kernels or young leaves (data not shown). Comparison of transcript levels for cv. Penawawa demonstrated reproducibility of data collected from separate experiments (Fig. 2A and B).

3.4. Characterization of recombinant wheat PPO antibody

A recombinant PPO construct of accession DQ217371 (constructed from accession BT009357 and engineered to

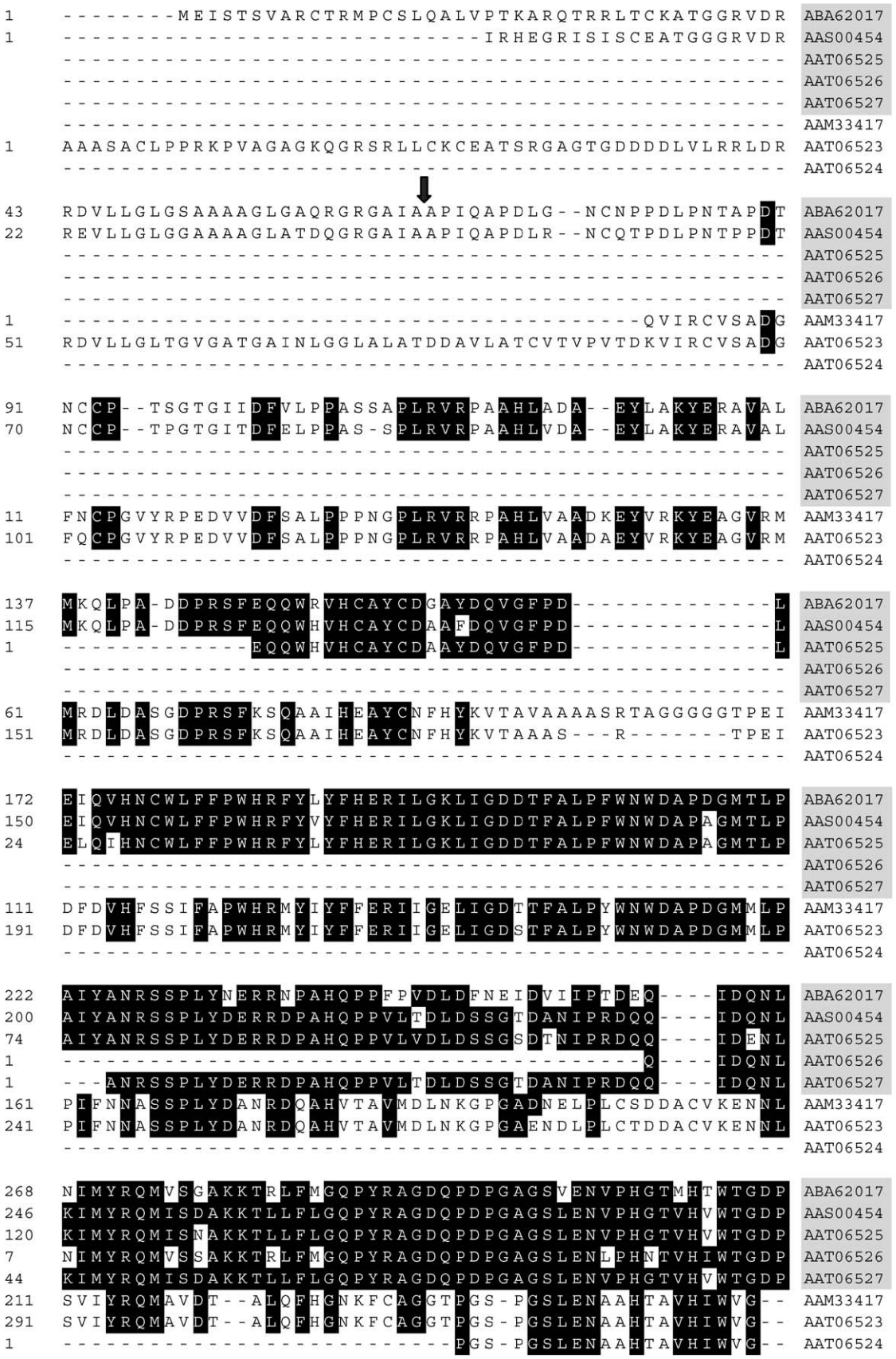

Fig. 1. Alignment of wheat PPO amino acid sequences obtained from GenBank that are classified either as putative kernel-specific (ABA62017, AAS00454, AAT06525, AAT06526, AAT06527; highlighted by gray background) or non-kernel-specific proteins (AAM33417, AAT06523, AAT06524). Gaps in sequence alignment are indicated (—). The black arrow indicates the predicted cleavage site of the chloroplast lumen transit peptide.

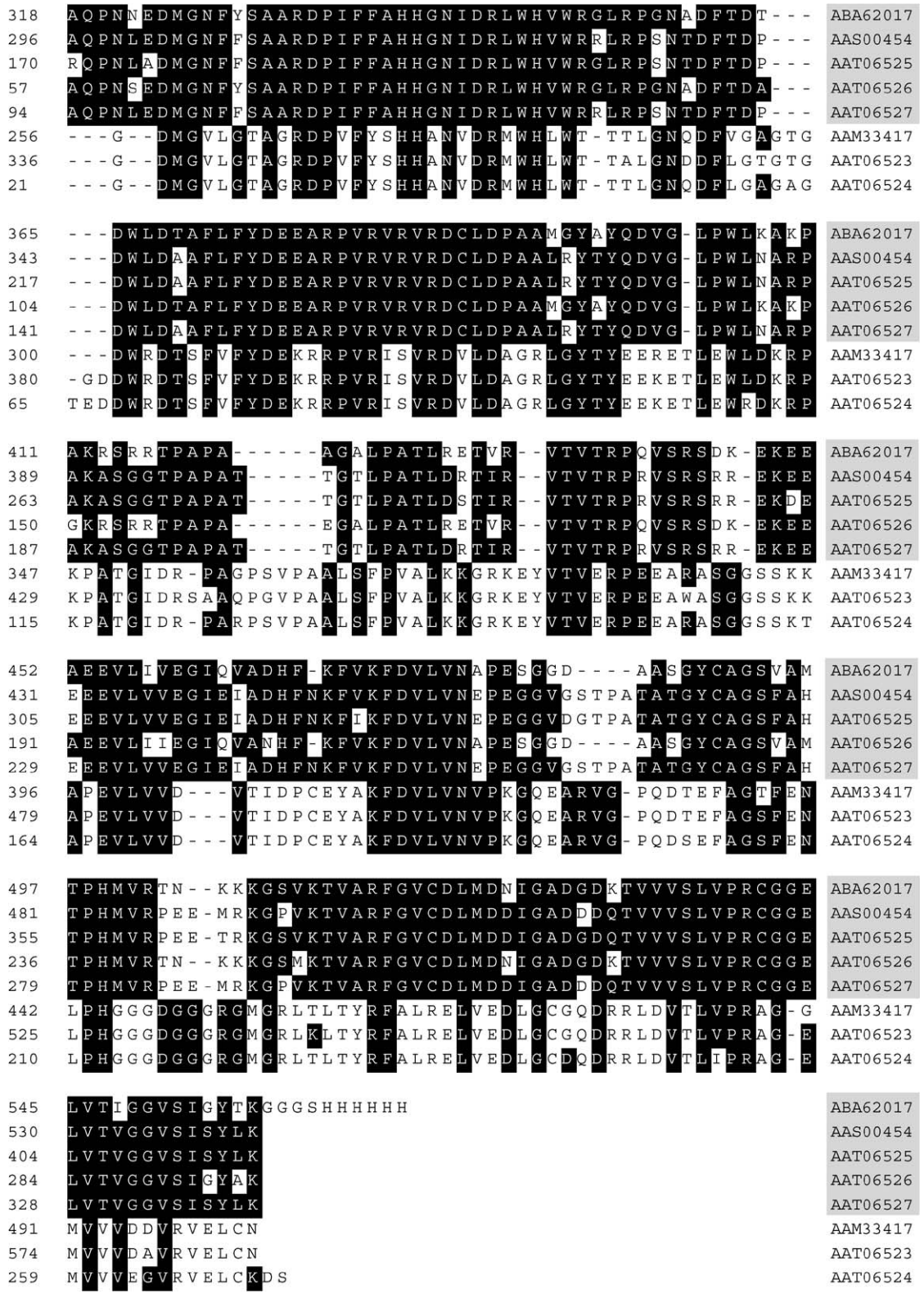

Fig. 1. (*Continued*)

contain a C-terminal 6X-His tag) was used to produce recombinant protein with an estimated size of ~63.9 kD (Fig. 3) which is close to the predicted size of 62.4 kD. LC-MS/MS analysis of the major peptide peaks, resulting from tryptic digestion of the recombinant protein, indicated exact amino acid sequence identity to the predicted sequence.

Immunoprecipitation experiments revealed that two wheat kernel proteins of ~60 and 62 kD could be resolved from both hexaploid (cv. Penawawa) and tetraploid

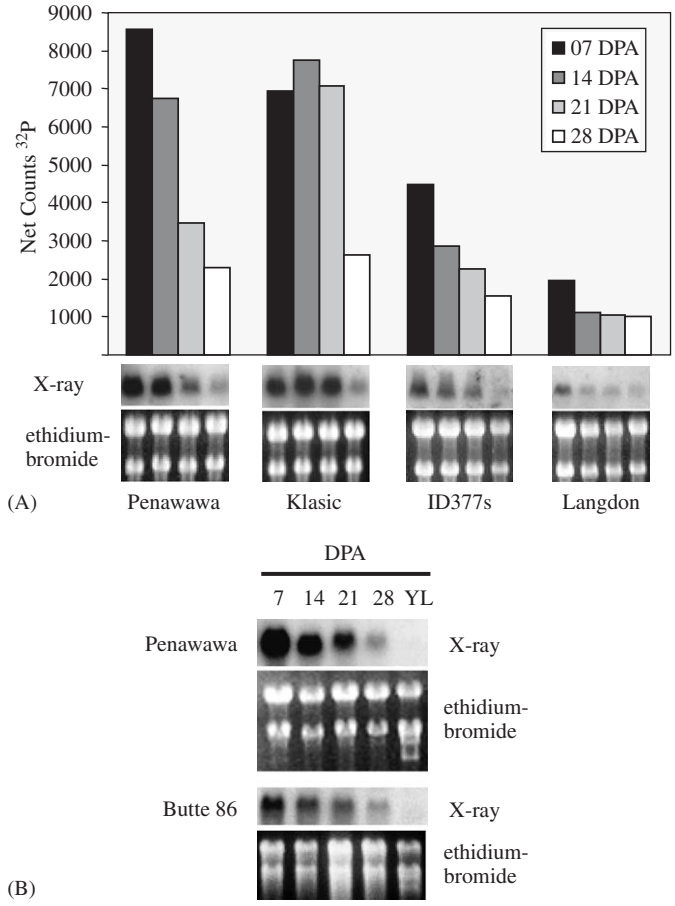

Fig. 2. Representative PPO transcript levels in wheat kernel or leaf tissue. Blots were probed with ^{32}P-labeled wheat PPO cDNA (accession BT009357). Histograms (A) above images represent net radioactivity. Representative transcript levels (B) in developing wheat kernels and young leaf tissue. Autoradiograph images indicate transcript levels at 7, 14, 21, and 28 DPA, or young leaf (YL) tissue (7 days after germination). All samples represent 10 μg total RNA lane^{-1} and all RNA blots were hybridized under identical conditions using equivalent X-ray film exposure times.

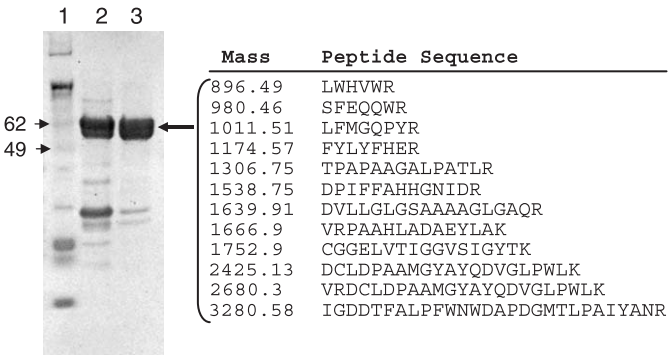

Fig. 3. Characterization of recombinant wheat PPO protein. SDS-PAGE profile of partially purified fractions of recombinant wheat PPO (lane 1, MW standards; lane 2, Ni-NTA affinity fraction; lane 3, size exclusion purification of affinity fraction). Proteins were stained with Coummassie Blue. Mass and predicted peptide sequence obtained from the purified protein (arrow pointing to lane 3) were determined by LC-MS/MS analysis.

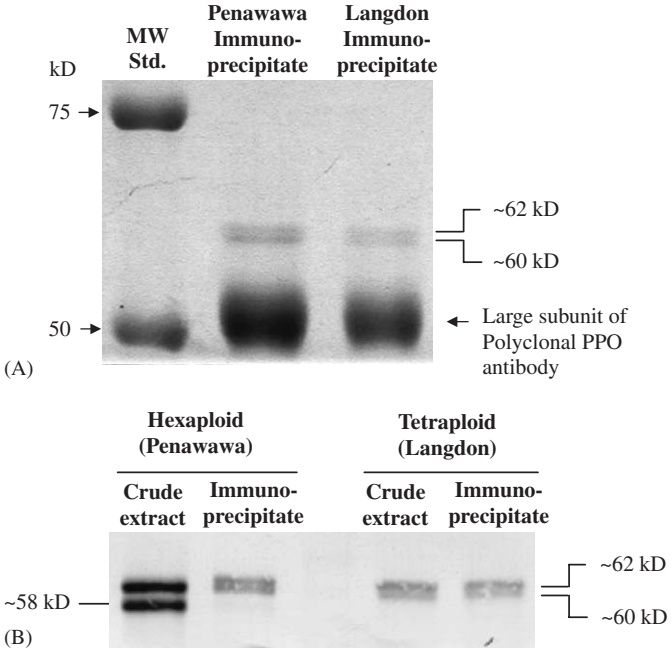

Fig. 4. Comparison of wheat kernel proteins immunoprecipitated from two cultivars with different PPO activity and ploidy levels. Commassie Blue-stained proteins (A) immunoprecipitated from 21-DPA kernel extract with wheat polyclonal PPO antibody. Western blot (B) of proteins of 21-DPA crude kernel protein extract (5 μg lane^{-1}) prior to or after immunoprecipitation with wheat polyclonal PPO antibody, probed with a 1:10,000 dilution of wheat PPO polyclonal antibody.

(cv. Langdon) wheat kernel protein extracts (Fig. 4A). Antigenic protein bands detected with wheat PPO antibody were similar in the Langdon crude extract and immunoprecipitate (Fig. 4B) but were dissimilar between the crude extract and immunoprecipitate of Penawawa. The Penawawa crude extract contained two major antigenic bands of ~58 and ~60–62 kD. The ~58 kD protein clearly did not immunoprecipitate. It is also possible that the major ~60–62 kD protein from Penawawa crude extract did not immunoprecipitate; this dark-staining protein band may have masked the two lighter-staining immunoprecipitated proteins observed in Fig. 4A and B.

Sequence analysis of the two immunoprecipitated wheat proteins (Fig. 4B), from both hexaploid and tetraploid wheat, confirmed their identity as PPOs (Table 1). The ~60 and 62 kD PPOs of Penawawa have the best predicted sequence identity to putative kernel-specific wheat PPO accessions AAT06526 and AAS00454, respectively. The ~60 and 62 kD proteins of Langdon both have the best predicted sequence identity to putative kernel-specific wheat PPO accession AAT06526. Based on the sequence data presented in Table 1, it appears that the ~60 kD immunoprecipitated PPO protein of cv. Penawawa results from a different gene locus than the ~62 kD PPO protein, and is most likely not an artifact of protein degradation or proteolysis. However, the two immunoprecipitated proteins of cv. Langdon gave the best match

Table 1
Matching peptide sequences from Penawawa and Langdon 62 and 60 kD proteins

Sample[a]	Log (e)[b]	Mass + h[c]	Delta[d]	z[e]	Matching peptide sequences	Accession[f]
P-62 kD	−1.6	1011.508	−0.013	2	kktr[22]LFMGQPYR[29]agdq	AAT06526
	−8.1	2638.258	0.0073	3	vrvr[124]VRDCLDPAAMGYAYQDVGLPWLK[146]akpg	
	−2.6	2383.089	0.019	3	vrvr[126]DCLDPAAMGYAYQDVGLPWLK[146]akpg	
	−1.8	1043.596	0.0075	2	tvrv[175]TVTRPQVSR[183]sdke	
	−3.9	2780.208	0.019	3	kfvk[211]FDVLVNAPESGGDAASGYCAGSVAMTPH[238]mvrt	
	−7.7	2677.311	0.018	3	tvar[255]FGVCDLMDNIGADGDKTVVVSLVPR[279]cgge	
	−6.2	1726.720	0.014	2	tvar[255]FGVCDLMDNIGADGDK[270]tvvv	
P-60 kD	−5.1	1652.912	0.028	3	splr[93]VRPAAHLVDAEYLAK[107]yera	AAS00454
	−1.6	1207.684	0.019	2	dakk[259]TLLFLGQPYR[268]agdq	
	−1.5	1538.750	0.026	3	saar[312]DPIFFAHHGNIDR[324]lwhv	
	−3.0	1213.587	0.026	2	rdpi[315]FFAHHGNIDR[324]lwhv	
	−4.5	1695.849	0.029	2	aalr[374]YTYQDVGLPWLNAR[387]paka	
	−8.3	1854.956	0.030	2	rpak[391]ASGGTPAPATTGTLPATLDR[410]tirv	
L-62 kD	−2.6	1027.508	0.017	2	kktr[22]LFMGQPYR[29]agdq	AAT06526
	−1.9	1538.750	0.032	3	saar[73]DPIFFAHHGNIDR[85]lwhv	
	−3.0	1213.587	0.032	2	rdpi[76]FFAHHGNIDR[85]lwhv	
	−4.9	1364.754	0.041	2	rsrr[156]TPAPAEGALPATLR[169]etvr	
	−1.5	1043.596	0.020	2	tvrv[175]TVTRPQVSR[183]sdke	
	−6.7	2693.311	0.102	3	tvar[255]FGVCDLMDNIGADGDKTVVVSLVPR[279]cgge	
	−7.0	1726.720	0.066	2	tvar[255]FGVCDLMDNIGADGDK[270]tvvv	
L-60 kD	−2.0	1011.508	0.027	2	kktr[22]LFMGQPYR[29]agdq	AAT06526
	−1.9	1538.750	0.054	3	saar[73]DPIFFAHHGNIDR[85]lwhv	
	−3.6	1213.587	0.044	2	rdpi[76]FFAHHGNIDR[85]lwhv	
	−2.6	3105.423	0.120	3	hvwr[92]GLRPGNADFTDADWLDTAFLFYDEEAR[118]pvrv	
	−5.8	2638.258	0.107	3	vrvr[124]VRDCLDPAAMGYAYQDVGLPWLK[146]akpg	
	−4.0	1364.754	0.048	2	rsrr[156]TPAPAEGALPATLR[169]etvr	
	−8.0	2677.311	0.114	3	tvar[255]FGVCDLMDNIGADGDKTVVVSLVPR[279]cgge	
	−5.4	1726.720	0.085	2	tvar[255]FGVCDLMDNIGADGDK[270]tvvv	
	−4.4	1666.826	0.073	2	lvpr[280]CGGELVTVGGVSIGYAK[296]	

Wheat kernel proteins were immunoprecipitated with an antibody to a recombinant wheat PPO protein. Sequences are from database searches of LC-MS/MS data (flanking sequences are in lower case). Underlined residues indicate peptides that have mass increases of 57 due to alkylation of C and/or 16 due to oxidation of M.

[a]P, Penawawa; L, Langdon.
[b]Log (e), probability of finding a match randomly.
[c]Mass + h, protonated peptide mass.
[d]Delta, deviation observed from matched.
[e]z, charge of identified peptide.
[f]Accession, GenBank protein accession designation.

to a single accession, either because they represent two post-translational products of a single gene, or because they are products of two similar genes. Data presented in Table 1 clearly confirm that antibody developed to recombinant PPO protein preferentially immunoprecipitated protein with predicted amino acid sequences most similar to kernel-specific wheat PPOs shown in Fig. 1.

3.5. Characterization of antigenic wheat kernel proteins and PPO activity

Antigenic PPO protein was monitored at different developmental stages using the polyclonal wheat PPO antibody. The highest levels of extractable antigenic proteins generally occurred from 7 to 28 days post-anthesis (DPA) and declined substantially by physiological maturity (35 DPA) in all cvs. (Fig. 5A). Crude extracts from hexaploid wheat (cvs. Penawawa and Klasic) with high kernel PPO activity and poor noodle color traits (Fuerst et al., 2006b; Morris et al., 2000) had two major antigenic protein bands at ~58 and ~60–62 kD (Fig. 5A). In comparison, the hexaploid cv. ID377s, with lower kernel PPO activity and possessing superior Asian noodle color traits, had reduced levels of the same two protein bands (Fig. 5A). The antigenic ~58 kD protein had reduced staining intensity in the low PPO cv. ID377s and was absent in the near-zero PPO tetraploid cv. Langdon (Fig. 5A). With longer substrate color development, a faint protein band of ~75 kD was observed from 7 to 21 DPA (Fig. 5B) which may represent a precursor PPO containing a transit peptide previously estimated to be 14–16 kD (Anderson and Morris, 2003b). Under high protein loads, antigenic proteins of ~62 and 75 kD were also observed in seedling leaves of Penawawa (Fig. 5C). The majority of

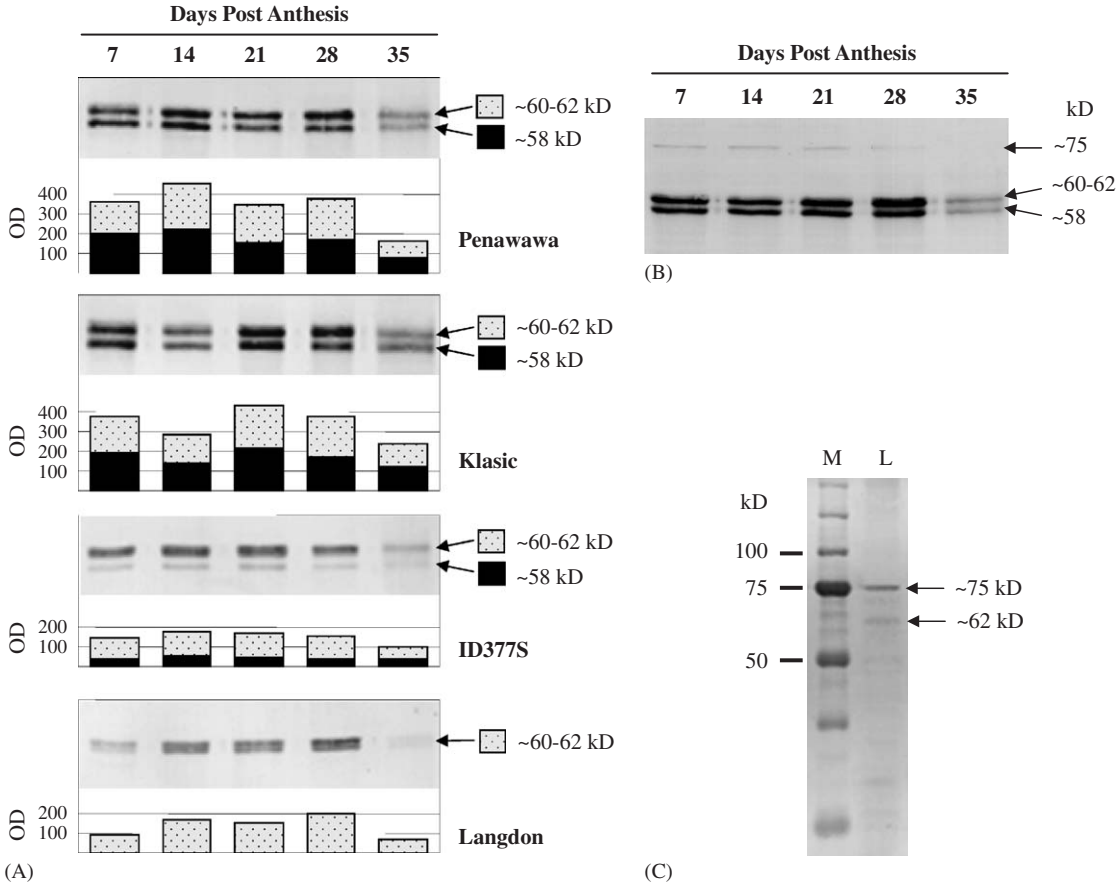

Fig. 5. Comparison of developing wheat kernel protein antigenic to polyclonal wheat PPO antibody. Western blot (A) of wheat kernel protein (5 μg lane^{-1}) from cultivars with varying levels of PPO activity separated by 7.5% SDS-PAGE, transblotted to PVDF and probed with a 21:10,000 dilution of wheat PPO polyclonal antibody. Arrows indicate major antigenic protein bands. Histograms shown (A) below each Western blot represent the optical density (OD) of the ~58 and ~60–62 kD bands over the course of seed maturation. Replicate of cv. Penawawa (B), as shown in (A), but separated by 10% SDS-PAGE and given longer development time to enhance the faint protein band at ~75 kD. Western blot (C) of wheat seedling crude protein extract (50 μg) from leaf tissue of cultivar Penawawa; M, molecular weight markers; L, leaf tissue.

antigenic protein in wheat leaves appeared to be associated with the putative PPO precursor.

The quantity of PPO protein observed during kernel maturation (Fig. 5) does not appear to be closely correlated with the extractable PPO activity (Fig. 6A). The specific activity of extractable PPO protein increased during kernel maturation in the three hexaploid cvs. but decreased in cv. Langdon. The increase in specific activity of PPO during kernel maturation in hexaploid cvs. was not a result of reduced protein content, since total extractable protein increased during kernel maturation (Fig. 6B). However, the reduction in PPO specific activity of cv. Langdon may be the result of increased protein content during kernel maturation. PPO activity during maturation was also calculated as a function of kernel dry weight (see Fig. 6C for dry weight/fresh weight ratios), which resulted in similar trends to that observed in Fig. 6A (data not shown). The level of PPO activity observed in cv. Penawawa may be due to increased activation, since Penawawa had the lowest PPO specific activity at 21 DPA but very high PPO specific activity in mature seeds (Fig. 6A). A similar trend was observed in other experiments, in which Penawawa PPO specific activity increased >5-fold during maturation, while cv. ID377s (low PPO) specific activity increased <3-fold (Table 2). The decreasing PPO specific activity in cv. Langdon (Fig. 6A) suggests that this cv. may lack a PPO activation mechanism.

Diphenol (L-DOPA) oxidation in wheat kernels was specifically attributed to PPO based upon sensitivity to PPO inhibitors such as tropolone (Fuerst et al., 2006b). We have further evaluated sensitivity of L-DOPA oxidation to tropolone and heat treatment in immature vs. mature kernels in order to assess the role of PPO (Table 2). Tropolone caused nearly 100% inhibition in all mature kernel assays, implying that this activity was due to PPO. However, tropolone inhibition was reduced in immature kernels, particularly for the 30-min duration; thus, L-DOPA oxidation in immature kernels may be partially attributed to a non-PPO mechanism, either enzymatic or non-enzymatic. This conclusion must be somewhat qualified due to the possible presence of a PPO isoform that is not completely inhibited by tropolone. For example, 1 mM tropolone partially inhibited PPO from eggplant (91% inhibition) (Perez-Gilabert and Garcia-Carmona, 2000)

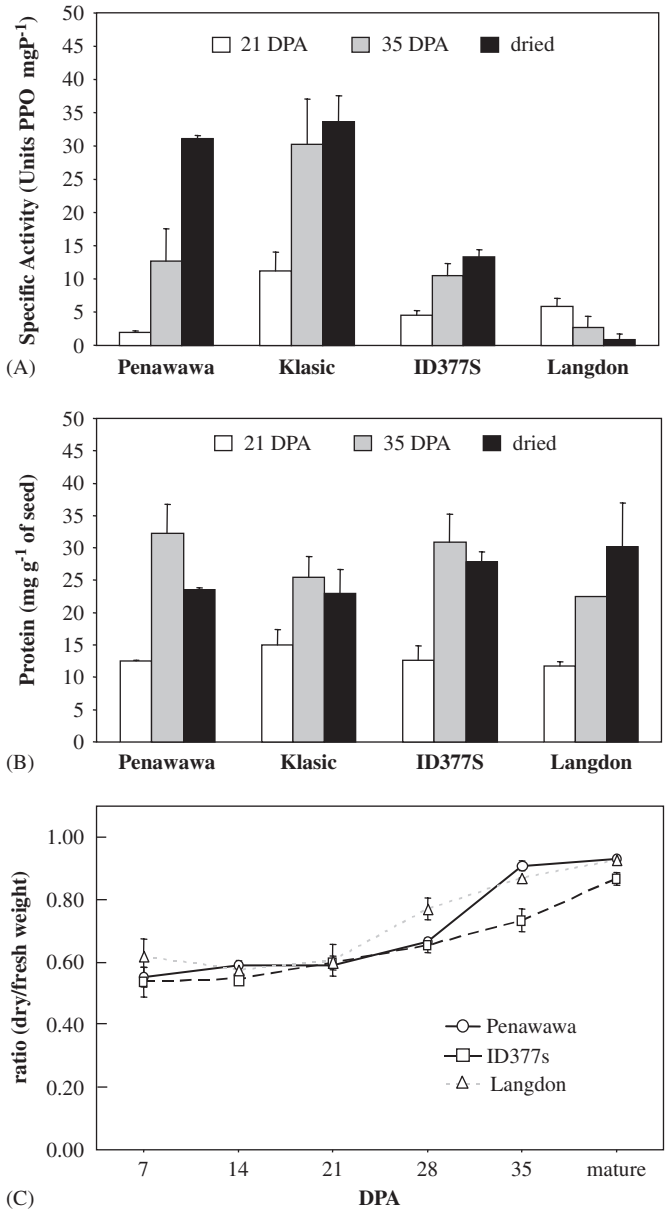

Fig. 6. PPO specific activity (A) and extractable protein (B) obtained from crude protein extracts of hexaploid (cvs. Penawawa, Klasic, ID377s) and tetraploid (cv. Langdon) wheat kernels during various stages of maturation. For comparison, the ratio of dry/fresh weight of cvs. Penawawa, ID377s, and Langdon are shown (C); "mature" = "dry". Individual bars represent the mean value of three technical replicates; error bars represent standard deviations.

and banana (80 to 95%) (Sojo et al., 1998). As in the case of tropolone, inhibition by heat treatment was generally lower in the 30-min duration assays (Table 2). Incomplete inhibition by heat treatment can be attributed to non-enzymatic oxidation and/or to incomplete inactivation by heat. PPO may be somewhat heat-tolerant, since PPO activity was only inhibited 53–76% when wheat kernels were incubated at 100 °C for 12 min (Vadlamani and Seib, 1996). It is likely that our heat treatment also did not completely inhibit PPO since tropolone completely inhibited L-DOPA oxidation in mature kernel extracts, yet, in the same extracts, heat treatment caused slightly less inhibition, 96% and 91% inhibition in Penawawa and ID377s, respectively, in the 30-min duration assays. This observation is most likely best explained as incomplete inhibition by the 5-min 100 °C incubation. In summary, our results confirmed that L-DOPA oxidation in mature kernels is due to PPO. In addition, immature kernels oxidize L-DOPA via both PPO and a non-PPO mechanism and/or a tropolone-insensitive PPO.

3.6. Comparison of whole-kernel assays and antigenic proteins among wheat lines

Kernel PPO assays (AACC, 2000; Anderson and Morris, 2001) have been developed as a rapid indicator for identifying cvs. with elevated PPO activity and have also been used to identify QTLs for PPO in wheat (Demeke et al., 2001; Raman et al., 2005). Substitution of the hexaploid 2D chromosome for the corresponding 2A or 2B chromosome of tetraploid wheat (N2A/D2D and N2B/D2D, respectively) indicated that elevated kernel PPO activity is clearly associated with the 2D chromosome (Fig. 7).

The association of antigenic protein bands of ~58 and ~60–62 kD with specific genomes and chromosomes appears to be complex. The antigenic ~58 kD protein was initially observed in hexaploid but not tetraploid wheat (Fig. 5); however, analysis of several aneuploid lines indicates that expression of this protein is not specifically correlated with the presence of chromosome 2D. For example, the ~58 kD protein was absent in all three tetraploid lines, Langdon, N2A/D2D, and N2B/D2D (Fig. 8A). In addition, while cv. Chinese Spring (donor of the D-genome chromosomes for Langdon substitution lines) and all hexaploid wheat lines contained the antigenic ~58 kD protein (Fig. 8A), chromosome 2D nullisomic/tetrasomic lines (C.S. N2D/T2A and C.S. N2D/T2B) actually contained higher levels of the ~58 kD protein than parent cv. Chinese Spring (Fig. 8A). Therefore, among the lines we have evaluated, the abundance of the ~58 kD protein appeared to require a hexaploid wheat background but not chromosome 2D. In contrast, C.S. N2B/T2D, having no 2B chromosomes and an extra pair of 2D chromosomes, had similar levels of the ~58 kD protein but substantially increased levels of antigenic ~60–62 kD protein compared to the parent cv. Chinese Spring (Fig. 8A). Likewise, N2A/D2D and N2B/D2D lines had increased levels of ~60–62 kD protein compared to the parent cv. Langdon. These results suggest that a ~60–62 kD antigenic protein is enhanced, and possibly encoded, by the 2D chromosome.

The presence of the hexaploid 2D chromosome in disomic substitution lines of tetraploid wheat (N2A/D2D and N2B/D2D) increased PPO activity in kernel assays compared to the parent cv. Langdon (Fig. 8B). Similarly, deletion of the 2D and addition of an extra pair of 2A or

Table 2
Effect of tropolone (1 mM) and heating (incubated 5 min at 100 °C) on L-DOPA oxidation activity in extracts from immature (21 DPA) and mature (dry) wheat kernels

Cultivar	Assay duration (min)	Kernel type	Activity	% Inhibition	
			untreated	Tropolone	Heated
Penawawa	2	Immature	14.6±3.6[a]	90	100
		Mature	86.5±8.4[a]	98	97
	30	Immature	146±14.5[b]	68	85
		Mature	776±51[b]	100	96
ID377s	2	Immature	15.7±3.4[a]	100	100
		Mature	40.3±12.1[a]	100	100
	30	Immature	176±11.8[b]	91	98
		Mature	424±16.1[b]	100	91

Activity is reported as either specific activity (2 min assay) or total activity (30 min assay).
[a]Specific activity (units L-DOPA oxidation min^{-1} $mg\,P^{-1}$).
[b]Total activity (units L-DOPA oxidation after 30 min $mg\,P^{-1}$).

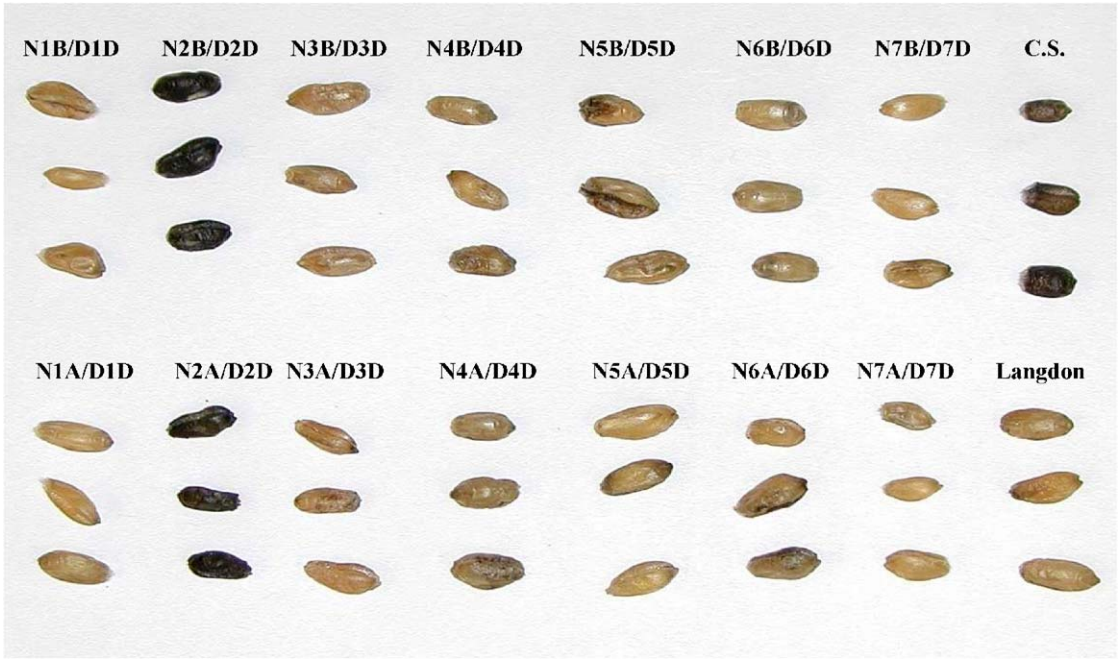

Fig. 7. Comparison of disomic substitution lines of Langdon wheat kernels. Wheat kernels were incubated 4 h in 10 mM L-DOPA. C.S., Chinese Spring hexaploid parent source of D chromosome; Langdon, tetraploid parent. Chromosome substitutions are abbreviated, for example, as N2B/D2D for nullisomic-2B/disomic-2D.

2B chromosomes, in C.S. N2D/T2A or C.S. N2D/T2B reduced activity in kernel assays compared to the parent Chinese Spring (Fig. 8B). The presence of a second pair of 2D but no 2B chromosomes (C.S. N2B/T2D) in hexaploid wheat resulted in PPO activity that was similar to the parent cv. Chinese Spring. Therefore, the presence of the 2D chromosome was generally well-correlated with kernel PPO activity and with the intensity of a ~60–62 kD protein band but not with the ~58 kD protein band. It is noteworthy, however, that cv.s Penawawa and Klasic had similar PPO activity levels (Fig. 8B), while the staining intensity of both protein bands was lower in Penawawa than Klasic (Fig. 8A). The discrepancy may be related to PPO latency and activation, as previously discussed.

3.7. PPO activity in immature and mature kernels

Immature kernels (7–21 DPA) of both tetraploid and hexaploid wheat contain more antigenic PPO protein than mature kernels (35 DPA) (Fig. 5A). In contrast, PPO activity in immature (21 DPA) kernels was substantially lower than that of mature kernels (Fig. 6A). Under non-denaturing conditions, PPO activity staining in SDS-PAGE gels appeared, at first, to be greater in immature kernels compared to mature kernels (Fig. 9A). However,

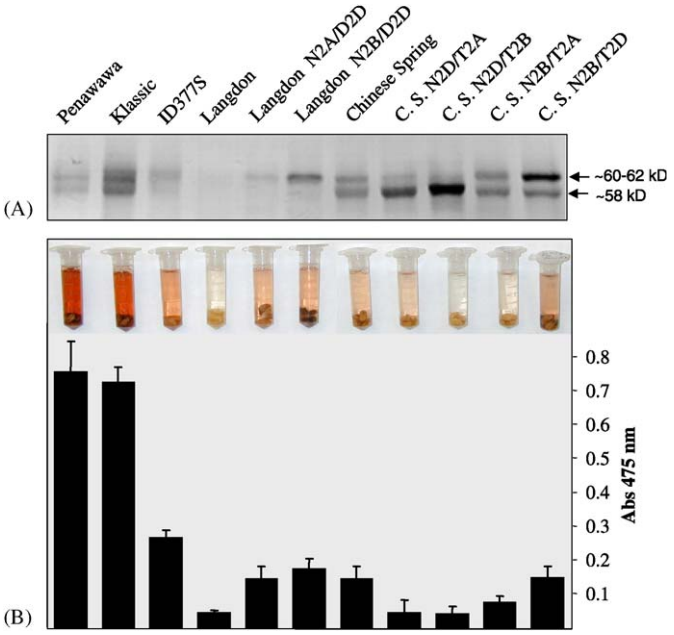

Fig. 8. Comparison of antigenic proteins from crude protein kernel extracts (A) vs. whole-kernel PPO activity (B) in hexaploid, tetraploid, and aneuploid wheat lines. Kernel protein (5 μg lane^{-1}) from crude extracts (A) were separated by 7.5% SDS-PAGE, blotted to PVDF and probed with a 1:10,000 dilution of wheat polyclonal PPO antibody. Whole-kernel PPO activity (B) was measured with five kernels per tube and representative sample tubes are shown; Abs 475 nm represents average absorbance of three biological replicates, each consisting of three technical replicate assays ($n = 9$); error bars represent standard deviations. Langdon substitutions abbreviated as in Fig. 7. Hexaploid lines are abbreviated, for example, as C.S. N2D/T2A for Chinese Spring nullisomic-2D/tetrasomic-2A.

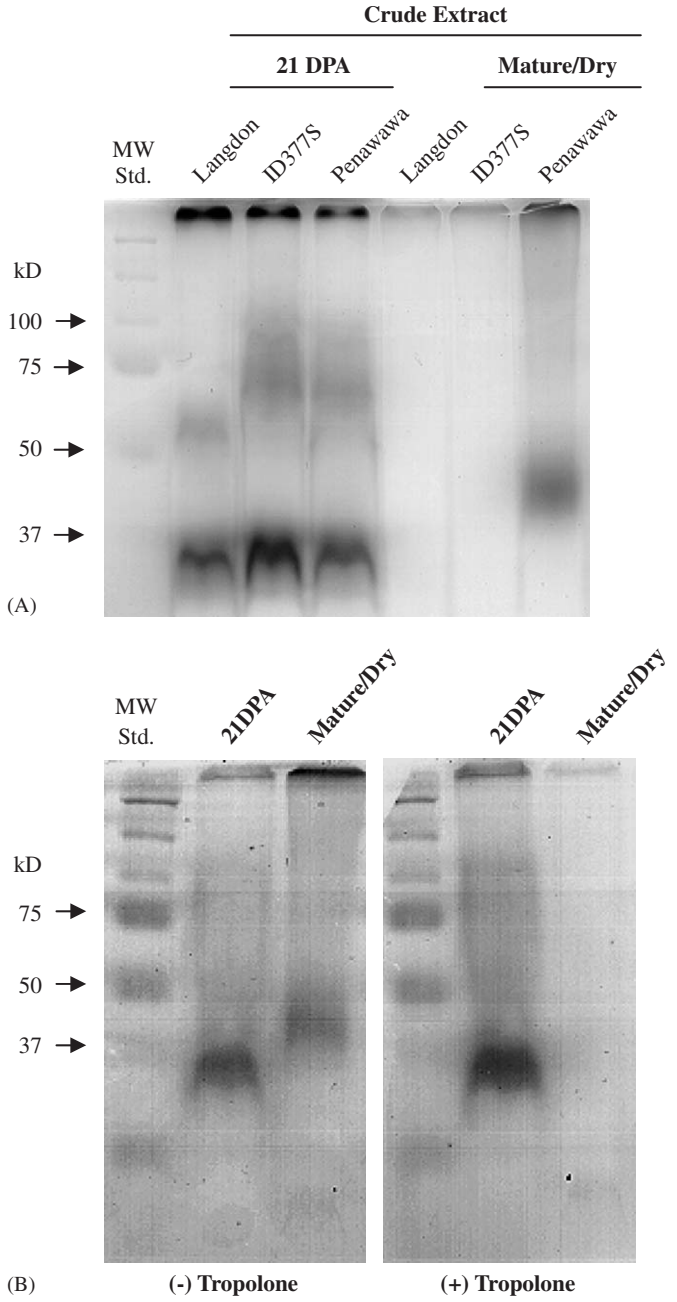

Fig. 9. SDS-PAGE assays for PPO activity in immature (21 DPA) and mature/dry kernels of hexaploid and tetraploid cultivars of wheat (A) or hexaploid wheat with and without pre-incubation in 1 mM tropolone (B). SDS-PAGE gels loaded with 150 μg crude kernel protein extract per lane; gels were incubated in 10 mM L-DOPA either with or without 1 mM tropolone for approximately 1–2 h.

pre-incubation of activity gels with 1 mM tropolone prior to incubation with L-DOPA did not inhibit the activity band observed at ~35 kD in lanes containing immature kernel extract (Fig. 9B). Pre-incubation of activity gels with tropolone did inhibit the ~45 kD band in lanes containing mature kernel extract (Fig. 9B), indicating that this activity band is most likely due to PPO. However, the ~35 kD band observed on activity gels may be due to L-DOPA oxidation by a non-PPO mechanism since this activity band did not show an antigenic reaction with our PPO antibody (data not shown). In addition, the activity band observed at ~45 kD in lanes containing mature kernel extracts (Fig. 9) corresponds to the ~45 kD PPO activity band previously reported for purified PPO from Penawawa (Anderson and Morris, 2003b).

4. Discussion

Discoloration of food products such as raw Asian noodles and steamed breads is a key determinant in consumer acceptance of wheat exported from the US, Canada, and Australia to wheat markets in Asia and the Middle East. PPO has been implicated as a key component leading to the discoloration phenomena (Fuerst et al., 2006b; Morris et al., 2000). Kernel assays developed to determine PPO levels early in the wheat breeding process indicated a positive correlation with chromosome 2D of hexaploid wheat (Anderson and Morris, 2001). Although the 2D chromosome appears to contribute to increased PPO activity in kernel assays, results obtained from this study are consistent with previous reports that other factors, such as PPO activation, contribute to total PPO activity.

Previous studies have indicated the presence of as many as 9–12 PPO isoforms in wheat (Kruger, 1976; Taneja et al., 1974) and PPO activity in bread wheat has been associated with genes located on several chromosomes (see Raman et al., 2005). Other recent studies, based on analysis of ESTs obtained from wheat sequencing projects, provide evidence that wheat PPOs are indeed composed of a multigene family (Jukanti et al., 2004) containing at least six PPO genes, of which three have been classified as kernel-specific. In this study, we identified cDNAs that best fit into the kernel-specific classification (Fig. 1). Antibody developed to the full-length coding region of one of the kernel-specific PPOs (Fig. 1, protein accession ABA62017) identified at least four wheat kernel proteins of ~58, 60, 62 and 75 kD in hexaploid wheat. Assuming that the ~75 kD wheat kernel protein is a precursor form of PPO containing a ~14–16 kD transit peptide required for plastid localization (Steffens et al., 1994), the remaining three wheat kernel PPOs observed in this study confirm the existence of at least three distinct kernel-localized PPO proteins.

Initial comparisons (Fig. 5) suggested that the ~58 kD kernel protein, showing antigenicity with antibody developed from a putative kernel-specific wheat PPO, was correlated with the level of PPO activity observed in four wheat cvs. Tetraploid durum (AABB) cv. Langdon used in this study clearly lacked the ~58 kD putative PPO isoform observed in hexaploid wheat (AABBDD), suggesting that it might be derived from the D genome. However, substituting hexaploid chromosome 2D for either chromosome 2A or 2B of tetraploid wheat (Fig. 8; Langdon N2A/D2D and Langdon N2B/D2D) or substituting an extra pair of 2D and removing 2B chromosomes (Fig. 8; C.S. N2B/T2D) had little effect on the ~58 kD protein relative to their respective parent cvs., but enhanced the amount of antigenic protein in the ~60–62 kD range. In contrast, removing chromosome 2D from hexaploid wheat and adding an extra copy of the 2A or 2B chromosome (Fig. 8; C.S. N2D/T2A and N2D/T2B) reduced the intensity of antigenic protein at ~60–62 kD and increased the intensity of the antigenic ~58 kD protein relative to the parent, cv. Chinese Spring. Based on the enhanced abundance of antigenic proteins at ~60–62 kD in the presence of chromosome 2D, it seems likely that one or more of these antigenic proteins originate from chromosome 2D, and unlikely that the antigenic protein at ~58 kD originates from chromosome 2D.

We observed a strong correlation between staining intensity at 60–62 kD and the presence of the 2D chromosome (Fig. 8). However, Langdon, which lacks the D genome, contained antigenic protein bands of ~60 and 62 kD. The antigenic protein band associated with chromosome 2D, therefore, probably represents a different protein. We previously suggested that Penawawa crude extract contained a major protein of ~60–62 kD that was unlike the two proteins in Langdon, since it may not have immunoprecipitated (Fig. 4B). We hypothesize that Penawawa, and wheat lines containing the 2D chromosome, contain a ~60–62 kD PPO isoform, distinct from those seen in Langdon, which was not clearly resolved on our gels. Further two-dimensional and proteomic analyses will be required to evaluate this hypothesis.

Compared to immature kernels, mature kernels had far lower antigenic protein staining intensity (Fig. 5) but higher PPO activity levels (Fig. 6A), indicating that PPO activation occurs during kernel maturation. PPO specific activity generally increased from 21 DPA to maturity; this apparent activation varied among cvs., with the greatest increase observed in cv. Penawawa (Fig. 6A, Table 2). However, the PPO of mature wheat kernels is still partially latent, since activation occurs in response to a wide range of detergents, solvents, and chaotropes (Fuerst et al., 2006a; Jukanti et al., 2003; Okot-Kotber et al., 2002), and in response to anion exchange purification, which increased total PPO activity several-fold (Anderson and Morris, 2003b). The biochemical basis for the variable latency in PPO activity is unknown but could involve conformational changes affecting inhibitor binding and/or access to substrate-binding sites (Steffens et al., 1994). The ~35 kD band observed on activity gels containing immature kernel extracts (Fig. 9) could not be attributed to PPO activity since it was not inhibited by tropolone, and did not react with our wheat PPO antibody. As a result, in this study, we were not able to demonstrate a conformational change in PPO during maturation to account for the tropolone-sensitive ~45 kD activity band observed in mature kernel extracts (Fig. 9). Further purification of PPO from immature kernels could help to characterize the activation process. In vivo proteolytic processing is also known to activate PPO in other species (Cho et al., 2003), but this may not be a factor during wheat kernel maturation, since the molecular weight of denatured antigenic proteins did not change during maturation (Fig. 5).

Although we saw kernel-specific expression of PPOs (Fig. 2) in this study as mentioned above, we observed no expression of a putative non-kernel-specific PPO transcript in wheat kernels or young leaves (data not shown). This contradicts a previous report indicating the identification of kernel transcript using a 760 bp fragment from accession AF507945 as a probe (Demeke and Morris, 2002), which we now believe belongs in the non-kernel PPO sub-class (Fig. 1, protein accession AAM33417). Since most non-kernel-specific cDNAs reported by Jukanti et al. (2004) came from libraries of anther or ovary tissue, it is tempting to suggest that this particular non-kernel specific PPO is expressed in other tissues. Recent studies have demonstrated tissue specificity and differential regulation of PPO transcripts for three red clover PPO genes, one of which was expressed in both petioles and flowers but not in leaf tissue (Sullivan et al., 2004).

Although the non-kernel-specific probe did not identify wheat transcripts in this study, our PPO antibody recognized proteins of ~62 and 75 kD in hexaploid wheat seedling leaves (Fig. 5C). However, this was only observed when 10-fold excess crude protein was loaded. It is not

surprising that this wheat PPO antibody reacted with PPOs in both leaves and kernels, since it also cross-reacted with red clover PPOs (GenBank accessions AAK13242, AAK13243, AAK13244) (data not shown) which only have 42–45% amino acid sequence identity with the recombinant PPO protein to which wheat PPO antibody was raised. Thus, the detection of wheat seedling PPO proteins does indicate that non-kernel PPOs are present in hexaploid wheat.

In summary, although kernel assays have been useful for identifying wheat breeding lines with high PPO activity, which appear to be partially associated with the 2D chromosome, data presented here suggest that other genetic factors, including PPO activation, also influence wheat kernel PPO activity, and consequently, wheat product discoloration. Thus, total PPO activity in mature kernels is most likely a function of the specific PPO isoforms present, their abundance, and their relative activation levels.

Acknowledgements

Special thanks to Scott Tingey, Maureen Dolan, and Petra Wolters (DuPont) for providing the wheat PPO clone (BT009357), to Brant Bigger and Laura Kelly (USDA/ARS) for technical help, and to Mike Sullivan (USDA/ARS) for providing plant and transgenic PPO proteins. This research was supported in part by US Department of Agriculture NRI Grant Award 2002-35503-12670.

References

AACC, 2000. Method 22–85. In: Approved Methods of the American Association of Cereal Chemists, 10th ed. The Association, St. Paul, MN.

Altschul, S.F., Madden, T.L., Shaffer, A.A., Zang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3402.

Anderson, J.V., Davis, D.G., 2004. Abiotic stress alters transcript profiles and activity of enzymes involved in glutathione-metabolism in *Euphorbia esula*. Physiologia Plantarum 120, 421–433.

Anderson, J.V., Morris, C.F., 2001. An improved whole-kernel assay for screening wheat germplasm for polyphenol oxidase activity. Crop Science 41, 1697–1705.

Anderson, J.V., Morris, C.F., 2003a. Characterization and expression of polyphenol oxidase in developing grain of wheat (*Triticum aestivum* L.). In: American Society of Plant Biologists Annual Meeting, Final Program and Abstract Supplement, p. 132. Available at 〈http://abstracts.aspb.org/pb2003/public/P45/0213.html〉.

Anderson, J.V., Morris, C.F., 2003b. Purification and analysis of wheat grain polyphenol oxidase protein. Cereal Chemistry 80, 135–143.

Anderson, J.V., Delseny, M., Fregene, M.A., Jorge, V., Mba, C., Lopez, C., Restrepo, S., Soto, M., Piegu, B., Verdier, V., Cooke, R., Tohme, J., Horvath, D.P., 2004. An EST resource for cassava and other species of Euphorbiaceae. Plant Molecular Biology 56, 527–539.

Bertrand, G., Muttermilch, W., 1907. Sur l'existence d'une tyrosinase dans le son de furmet. [On the presence of a tyrosinase in the wheat bran.] Comptes Rendus Hebdomadaires des Seances de l'Academie des Sciences 144, 1285.

Boehm, A.M., Galvin, R.P., Sickmann, A., 2004. Extractor for ESI quadrupole TOF tandem MS data enabled for high throughput batch processing. BMC Bioinformatics 5 (162) available at http://sourceforge.net/projects/protms/.

Chang, S., Puryer, J., Cairney, J., 1993. A simple and efficient method for isolating RNA from pine trees. Plant Molecular Biology Reporter 11, 113–116.

Cho, M.H., Moinuddin, S.G.A., Helms, G.L., Hishiyama, S., Eichinger, D., Davin, L.B., Lewis, N.G., 2003. (+)-Larreatricin hydroxylase, an enantio-specific polyphenol oxidase from the creosote bush (*Larrea tridentata*). Proceedings of the National Academy of Science USA 100, 10641–10646.

Craig, R., Beavis, R.C., 2004. Tandem: matching proteins with mass spectra. Bioinformatics 20, 1466–1467.

Demeke, T., Morris, C.F., 2002. Molecular characterization of wheat polyphenol oxidase (PPO). Theoretical and Applied Genetics 104, 813–818.

Demeke, T., Morris, C.F., Campbell, K.G., King, G.E., Anderson, J.A., Chang, H.-G., 2001. Wheat polyphenol oxidase: distribution and genetic mapping in three inbred line populations. Crop Science 41, 1750–1757.

Fenyo, D., Beavis, R.C., 2003. A method for assessing the statistical significance of mass spectrometry-based protein identifications using general scoring schemes. Annals of Chemistry 75, 768–774.

Fuerst, E.P., Anderson, J.V., Morris, C.F., 2006a. Polyphenol oxidase in wheat grain: whole-kernel and bran assays for total and soluble activity. Cereal Chemistry 83, 10–16.

Fuerst, E.P., Anderson, J.V., Morris, C.F., 2006b. Delineating the role of polyphenol oxidase in the darkening of alkaline wheat noodles. Journal of Agricultural and Food Chemistry 54, 2378–2384.

Gill, B.S., Friebe, B., Endo, T.R., 1991. Standard karyotype and nomenclature system for description of chromosome bands and structural aberrations in wheat (*Triticum aestivum*). Genome 34, 830–839.

Hunt, M.D., Eannetta, N.T., Yu, H., Newman, S.M., Steffens, J.C., 1993. cDNA cloning and expression of potato polyphenol oxidase. Plant Molecular Biology 21, 59–68.

Joppa, L.R., Williams, N.D., 1988. Langdon durum disomic substitution line and aneuploid analysis in tetrapliod wheat. Genome 30, 222–228.

Jukanti, A.K., Bruckner, P.L., Habernicht, D.K., Foster, C.R., Martin, J.M., Fischer, A.M., 2003. Extraction and activation of wheat polyphenol oxidase by detergents: biochemistry and applications. Cereal Chemistry 80, 712–716.

Jukanti, A.K., Bruckner, P.L., Fisher, A.M., 2004. Evaluation of wheat polyphenol oxidase genes. Cereal Chemistry 81, 481–485.

Kruger, J.E., 1976. Changes in the polyphenol oxidases of wheat during kernel growth and maturation. Cereal Chemistry 53, 201–213.

Mares, D.J., Campbell, A.W., 2001. Mapping components of flour and noodle colour in Australian wheat. Australian Journal Agricultural Research 52, 1297–1309.

Morris, C.F., Jeffers, H.C., Engle, D.E., 2000. Effect of processing, formula and measurement variables on alkaline noodle color-toward an optimized laboratory system. Cereal Chemistry 77, 77–85.

Morris, C.F., Massa, A., Anderson, J.V., Demeke, T., 2002. Progress in identification and selection of desirable genetic traits for wheat breeding and improvement. In: Black, C.K., Panozzo, J.F., Wrigley, C.W., Bately, I.L., Larsen, N. (Eds.), Cereals 2002 Proceedings of the 52nd Australian Cereal Chemistry Conference, The Royal Australian Chemical Institute Cereal Chemistry Division, pp. 227–230.

Nakayama, T., Yonekura-Sakakibara, K., Sato, T., Kikuchi, S., Fukui, Y., Fukuchi-Mizutani, M., Ueda, T., Nakao, M., Tanaka, Y., Kusumi, T., Nishino, T., 2000. Aureusidin Synthase: a polyphenol oxidase homolog responsible for flower coloration. Science 290, 1163–1166.

Newman, S.M., Eannetta, N.T., Yu, H., Prince, J.P., Carman de Vicente, M., Tanksley, S.D., Steffens, J.C., 1993. Organization of the tomato polyphenol oxidase gene family. Plant Molecular Biology 21, 1035–1051.

Okot-Kotber, M., Liavoga, A., Yong, K.-J., Bagorogoza, K., 2001. Activity and inhibition of polyphenol oxidase in extracts of bran and

other milling fractions from a variety of wheat cultivars. Cereal Chemistry 78, 514–520.

Okot-Kotber, M., Liavoga, A., Yong, K.-J., Bagorogoza, K., 2002. Activation of polyphenol oxidase in extracts of bran from several wheat (*Triticum aestivum*) cultivars using organic solvents, detergents, and chaotropes. Journal of Agricultural Food Chemistry 50, 2410–2417.

Perez-Gilabert, M., Garcia-Carmona, F., 2000. Characterization of catecholase and cresolase activities of eggplant polyphenol oxidase. Journal of Agricultural and Food Chemistry 48, 695–700.

Raman, R., Raman, H., Johnstone, K., Lisle, C., Smith, A., Matin, P., Allen, H., 2005. Genetic and in silico comparative mapping of the polyphenol oxidase gene in bread wheat (*Triticum aestivum* L.). Functional and Integrative Genomics 5, 185–200.

Sambrook, J., Fritsch, E.F., Maniatis, T., 1989. Molecular Cloning, A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press, New York, USA.

Simeone, R., Pasqualone, A., Clodoveo, L.M., Blanco, A., 2002. Genetic mapping of polyphenol oxidase in tetraploid wheat. Cellular and Molecular Biology Letters 7, 763–769.

Sojo, M.M., Nunez-Delicado, E., Garcia-Carmona, F., Sanchez-Ferrer, A., 1998. Partial purification of a banana polyphenol oxidase using triton X-114 and PEG 8000 for removal of polyphenols. Journal of Agricultural and Food Chemistry 46, 4924–4930.

Steffens, J.C., Harel, E., Hunt, M.D., 1994. Polyphenol oxidase. In: Ellis, B.E., Kuroki, G.W., Stafford, H.A. (Eds.), Recent Advances in Phytochemistry, Genetic Engineering of Plant Secondary Metabolism. Plenum Press, New York, pp. 275–312.

Sullivan, M.L., Hatfield, R.D., Thoma, S.L., Samac, D.A., 2004. Cloning and characterization of red clover polyphenol oxidase cDNAs and expression of active protein in *Escherichia coli* and transgenic alfalfa. Plant Physiology 136, 3234–3244.

Taneja, S.R., Abrol, Y.P., Sachar, R.C., 1974. Modulation of *o*-diphenolase and monophenolase enzymes during wheat development. Cereal Chemistry 51, 457–465.

Thygesen, P.W., Dry, I.B., Robinson, S.P., 1995. Polyphenol oxidase in potato. A multigene family that exhibits differential expression patterns. Plant Physiology 109, 525–531.

Vadlamani, K.R., Seib, P.A., 1996. Reduced browning in raw oriental noodles by heat and moisture treatment of wheat. Cereal Chemistry 73, 88–95.

Vensel, W.H., Tanaka, C.K., Cai, N., Wong, J.H., Buchanan, B.B., Hurkman, W.J., 2005. Developmental changes in the metabolic protein profiles of wheat endosperm. Proteomics 5, 1594–1611.

Vickers, J.E., Grof, C.P.L., Bonnett, G.D., Jackson, P.A., Knight, D.P., Roberts, S.E., Robinson, S.P., 2005. Overexpression of polyphenol oxidase in transgenic sugarcane results in darker juice and sugar. Crop Science 45, 354–362.

Whitaker, J.R., Lee, C.Y., 1995. Recent advances in chemistry of enzymatic browning: an overview. In: Lee, C.Y., Whitaker, J.R. (Eds.), Enzymatic Browning and its Prevention. American Chemical Society, Washington, DC, pp. 2–7.